# Exome Sequencing Identifies a Novel Pathogenic Variant in *RAB3GAP1* Causing Warburg Micro Syndrome in a Pakistani Family

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## Abstract

**Background:** Warburg Micro syndrome is a rare heterogeneous recessive genetic disorder characterized by ocular, neurological and endocrine problems. To date, disease causing variants in four genes have been identified to cause this syndrome; of these, *RAB3GAP1* variants are the most frequent. Very little is known about Warburg Micro syndrome in rural populations.

**Objectives:** This study aimed to investigate the genetics underpinnings of Warburg Micro syndrome in a Pashtun family with two patients from Pakistan. The patients presented with spastic diplegia, severe intellectual disability, microphthalmia, microcornea, congenital cataracts, optic atrophy and hypogonadism.

**Methods:** MRI analysis revealed pronounced cerebral atrophy including corpus callosum hypoplasia and polymicrogyria. Exome sequencing and subsequent filtering identified a novel homozygous missense variant NM_001172435: c.2891A>G, p.Gln964Arg in the *RAB3GAP1* gene. The variant was validated, and its segregation confirmed, by Sanger sequencing.

**Results:** Multiple prediction tools assess this variant to be damaging and structural analysis of the protein shows that the mutant amino acid residue affects polar contact with the neighboring atoms. It is extremely rare and is absent in all the public databases. Taken together, these observations suggest that this variant underlies Micro syndrome in our family and is extremely important for management and family planning.

**Conclusions:** Identification of this extremely rare variant extends the mutations spectrum of Micro syndrome. Screening more families, especially in underrepresented populations will help unveil the mutation spectrum underlying this syndrome.

**Keywords:** WARBM, RAB3GAP1, Micro syndrome, Rab18, Spastic diplegia

## Introduction

Warburg Micro syndrome (WARBM), sometimes referred to as Micro syndrome, is a heterogeneous autosomal recessive genetic disorder characterized by ocular, neurological and

endocrine problems. Typical symptoms of the disease include microcephaly, microphthalmia, microcornea, congenital cataracts, optic atrophy, corpus callosum hypoplasia, intellectual disability, spastic diplegia and hypogonadism[1]. These symptoms overlap with cerebro-oculo-facio-skeletal syndrome (MIM #214150), Cockayne syndromes A (MIM #216400) and B (MIM #133540), and Martsolf syndrome (MIM #212720)[3]. However, in the case of WARBM syndrome, intellectual disability is more severe and can be diagnosed by cranial MRI usually, which typically shows cortical dysplasia, in particular hypoplasia or agenesis of the corpus callosum. Understanding the genetics of rare disorders such as WARBM is essential for management and family planning.

WARBM syndrome was first reported in two patients of a consanguineous Pakistani family in 1993 and, though its true incidence remains unknown, it is extremely rare[1]. To date, pathogenic variants in four different genes, *RAB3GAP1* (RAB3 GTPase-Activating Protein Catalytic Subunit; MIM *602536; 2q21.3)[2], *RAB-3GAP2* (RAB3 GTPase-Activating Protein Noncatalytic Subunit; MIM *609275; 1q41)[4], *RAB18* (Ras-Associated Protein RAB18; MIM *602207; 10p21.1)[5] and *TBC1D20* (TBC1 Domain Family Member 20; MIM *611663; 20p13)[6], have been linked with WARBM syndrome. Each of these genes, when mutated, underlies a separate subtype, WARBM1, WARBM2, WARBM3 and WARBM4, respectively. Among these, mutations in *RAB3GAP1* are the most common, reported in as many as 70% of WARBM syndrome patients[7]. In this report, we present a novel missense pathogenic *RAB3GAP1* variant (NM_001172435: c.2891A>G) in a consanguineous Pakistani family of Pashtun ancestry, diagnosed with WARBM1.

## Methods

### *Ethical Approval*

The study was formally approved by the Institutional Bioethical Committee (IBC) of Islamia College University Peshawar (Ref. No. 602/ORIC/ICP). An informed written consent, for genetic analysis and publication of the results, was obtained from the parents after explaining the purpose and expected results of the study. The pedigree was drawn by interviewing the parents and other elder members of the family. Samples from the two affected individuals, their parents and a phenotypically healthy sibling were collected along with photographs and videos. Blood samples were drawn in EDTA tubes and stored at -20°C.

***Whole Exome Sequencing (WES)***

WES was performed on the DNA from the two affected individuals, using Illumina platform HiSeq 2500 systems (Illumina, San Diego, CA, USA) with an average coverage of 150x, covering approximately 97.5% of the target bases. All the sequence reads were assessed for quality check using FastQC to get quality reads. Reads were aligned to the reference human genome (GRCh38) using the Burrows Wheeler Aligner (BWA) tool and duplicates were removed using Picard. Variants were called using GATK in a variant calling file (VCF). Initially common and intronic variants were removed. All functional variants were prioritized for rare variants by filtering through public databases such as Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/). Only homozygous or compound heterozygous, non-synonymous, frameshift, splice site and coding indel variants with allelic frequencies of less than <0.001% in the 1000 genome project, dbSNP150 and gnomAD database were selected for further analysis. Functional annotation of the surviving variants was done using ANNOVAR (www.annovar.openbioinformatics.org).

***Sanger Sequencing***

The candidate variants were validated by Sanger sequencing. For segregation analysis, the variants were sequenced in all the available individuals including parents. Primers were designed using Primer 3 plus (https://bioinfo.ut.ee/primer3). Sanger sequencing was performed using Big Dye Terminator cycle sequencing kit (version 3.1, Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) and capillary electrophoresis on the 3730 DNA analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Only one variant (NM_001172435: c.2891A>G) was found segregating with the disease symptoms in the family (Forward primer: 5′-AGAGAATGGGCTCCCCAGAG-3′; Reverse primer: 5′-GAGGCAGCACTGTCTCTGAA-3′). Sequence data were analyzed chromatograms were generated using sequence analysis software Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA).

## Results

### *Clinical Features*

The proband (IV:2) was identified at the outdoor patient department of Lady Reading Hospital (LRH), Peshawar and, afterwards, sampled along with the rest of the family members at their home. The family was examined by a neurologist and an ophthalmologist at LRH, Peshawar. There were three affected patients in the family (Figure A) including a 23 years old girl (IV:1), a 22 years old boy (IV:2), and a deceased girl (IV:4) who died at 9 years of age. The patients were born at term by normal vaginal delivery. They were hypotonic in infancy, with poor head control, however, their failure to thrive was noticed at six months of age. At the time of examination, the two available patients present with cataract, microphthalmia and microcornea. They have severe intellectual disability, and paralysis from head down, including trunk, legs and arms. Paralysis is more severe in the male patient as compared to the female. They are both wheelchair bound, have short stature, scoliosis and

overlapping toes. Language acquisition is compromised and they cannot communicate effectively with family members including their parents. The male patient has rarefied eyebrows, maxillary protrusion and beaked nose. He has relatively small scrotum and a hypoplastic penis. The female has a less prominent maxillary protrusion but prominent nasal bridge and root (Figure B). As per her mother, her labia minora was smaller; however, the family did not consent for her clinical inspection. According to parents, phenotypes of the deceased patient were similar to the female patient (IV:1). Results of liver and kidney function tests were unremarkable. Magnetic resonance imaging (MRI) of the male patient revealed multiple atrophic changes in the brain and narrowed corpus callosum. There are atrophic changes in the fronto-parietal lobe and temporal lobe with small optic disc and a simplified pattern of sulci and gyri (Figure C). Ultrasonic studies confirmed a normal position of the kidneys and other organs in both patients. Biochemical assays, such as lipid profile, renal function test, liver function test and full blood count were also unremarkable.

***Genetic Analysis***

Whole exome sequencing was performed on the DNA of two patients (IV:1 and IV:2) and their mother (III:2) and a total of 106,009, 105,335 and 105,619 variants were obtained, respectively. Considering the recessive nature of the disease, inferred by pedigree analysis, and consanguinity in the family, we filtered the data for rare recessive variants with a minor allele frequency (MAF) of ≤0.01 in the dbSNP150, Exome Aggregation Consortium, gnomAD, 1000 Genomes Project. We identified one variant, *RAB3GAP1*:c.2870A>G, for which the two patients were homozygous whereas the mother was heterozygous. Sanger sequencing of all the family members validated the variant and confirmed its segregation in the second parent (III-1) and the phenotypically healthy sister (IV:3) (Figure D). We did not find any other segregating variant in the exome data. Pathogenicity of the variant

(NM_001172435: c.2891A>G, p.Gln964Arg) was assessed using different *in silico* prediction tools, such as SIFT (Damaging: 0.00), MutationTaster (Disease Causing), Polyphen2 (Probably Damaging; score: 0.912), Provean (Deleterious; score: -2.587), I-Mutant (Decreases stability), MUpro (Decreases stability) and Phd-SNP (Deleterious). We also assessed the predicted effect of the amino acid change on the stability of the mutant RAB3GAP1, using Expasy's ProtParam server (http://web.expasy.org/protparam/). With a reliability index of 2 (RI = 2), the replacement of Glutamine by Arginine is predicted to decrease the stability of the mutant protein (Table S1). Structure of the wild type and mutant RAB3GAP1 was constructed and analyzed using Modeller 9.19[8]. Multiple sequence alignment of human RAB3GAP1 homologous proteins showed that the amino acid residue at the position Glu964 is strictly conserved across vertebrates (Figure S2).

## Discussion

WARBM is a rare autosomal recessive disease characterized by ocular, neurologic and endocrine problems. It is a phenotypically and genetically heterogeneous syndrome caused by mutations in *RAB3GAP1*, *RAB3GAP2*, *RAB18*, and *TBC1D20*[2,4-6]. Mutations in any of these genes result in WARBM with clinically indistinguishable and overlapping symptoms. The *RAB3GAP1* encoded protein helps regulate the activity of GTPases, which are specialized proteins that control a variety of cellular functions. To perform its function, RAB3GAP1 and RAB3GAP2 form a complex known as the RAB3GAP complex. This complex activates a GTPase RAB18 by exchanging GTP for the attached GDP and inactivates another GTPase known as RAB3 by stimulating a reaction that turns the attached GTP into GDP. RAB18 is involved in the organization of endoplasmic reticulum and, hence, in protein processing and transport whereas RAB3 plays a role in the release of hormones and brain chemicals (neurotransmitters) from cells. Mutations in the RAB3GAP complex have also been reported

to cause Martsolf syndrome, a disease that has similar, albeit milder, symptoms as WARBM1[9]. The former presents with moderate intellectual disability and developmental delay, and longer life expectancy with less pronounced cerebral anomalies[10,11]. The milder phenotype in the case of Martsof syndrome is suggested to underlie variants that affect the function of RAB3GAP proteins but still allow some normal protein to be produced thereby ameliorating the clinical phenotype[12]. The disease symptoms of patients in the current study are compatible with WARBM1 because they present with severe intellectual disability. Brain MRI of the male patient (IV:2) revealed narrowed corpus callosum and multiple atrophic changes in the fronto-parietal lobe, temporal lobe, small optic disc and a simplified pattern of sulci and gyri (polymicrogyria and pachygyria) (Figure C). This pronounced cerebral atrophy is one of the characteristic features of WARBM 1. Both the living patients have severe developmental delay and postnatal failure to thrive, congenital bilateral cataracts and microcornea, general hypotonia and hypogonadism.

We observed an intra-familial clinical heterogeneity in the family; the female patient presents with relatively milder symptoms; she has less pronounced maxillary protrusion, normal eyebrows and contrary to her brother, she does not have a beaked nose but a prominent nasal bridge and root. According to the mother of the patients, clinical features of the deceased female patient were nearly identical to her sister (IV:1). None of the patients had microcephaly and large ears. Apart from this phenotypic heterogeneity, which is frequently reported in WARBM1 patients[3], most of the characteristic features of the micro syndrome were present in these patients.

Exome sequencing, followed by Sanger validation, identified a missense variant (NM_001172435: c.2891A>G, p.(Gln964Arg)) in *RAB3GAP1*, which segregates recessively in the family. Mutations in *RAB3GAP1* are the most common cause of Micro syndrome.

Mutations in this gene leads to RAB18 deficiency, which, subsequently, affects eyes, brain and reproductive system[13,14]. Missense mutations in this gene have been previously associated with WARBM1[9,15]. *In vitro* assessment of two missense variants in RAB3GAP1, p.Thr18Pro and p.Glu24Val, suggests that point mutations at conserved residues of the RAB3GAP1-RAB3GAP2 complex result in loss of the Rab18 GEF and membrane-targeting activities[16]. Similarly, frameshift variants located in the last exon of *RAB3GAP1* (c.2865_2868insTTCT, p.Pro955Serfs*15 and c.2801delC, p.Pro934Leufs*87) have been reported to cause Micro syndrome. Since it is unlikely that transcripts carrying these variants is will be subject to NMD, the extreme C-terminal domain of the RAB3GAP1 seems essential for protein function or stability[17].

The variant reported in the current study is extremely rare and was not found in any of the public databases (gnomAD, 1000 Genomes, GenomeAsia, Mexican DB, Iranome and GME Variome) even in heterozygous state (Accessed on June 29, 2022). *In silico* tools predict that this variant has a deleterious effect on the protein function and decreases its stability (RI = 2) (Table S1). Structure analysis reveals a difference in the orientation of the wild type (Gln964) and mutant (Arg964) RAB3GAP1 protein. The polar contact of both the residues also varies; whereas Gln964 makes 9 polar contacts, Arg964 makes 6 contacts. This bonding difference is affecting the nearby chains (Figure S1). We also noticed that the residue p.Gln964 appears to be in close proximity to the residue changed by a previously identified pathogenic variant, p.Arg187 (Figure S3).

According to the guidelines of American College of Medical Genetics and Genomics[18], we classified this variant as likely pathogenic, because i) it is absent from population databases (PM2), ii) it co-segregates with WARBM in two of affected individuals in the family (PP1), iii) multiple *in silico* tools predicted this variant to be disease causing or deleterious (PP3)

and iv) the phenotype is very specific for *RAB3GAP1* (PP4). Moreover, multiple sequence alignment of human RAB3GAP1 homologous proteins shows that amino acid Arg964 lies in a stretch of six amino acids (Leu962-Met966) that are strictly conserved in evolution across vertebrates (Figure S2). Combining these evidences and observation, we believe that the variant *RAB3GAP1*:c.2891A>G, p.(Gln964Arg) is deleterious for the protein and is responsible for Warburg Micro syndrome in this family.

## Conclusions

In conclusion, we identified a novel missense variant (c.2891A>G, p.(Gln964Arg)) in *RAB3GAP1* gene in two individuals of a consanguineous Pakistani family affected with Warburg Micro syndrome. We checked co-segregation of this mutation in non-symptomatic family members and concluded that the disease phenotype segregate with a homozygous genotype. Our findings expand the spectrum of genetic mutations in the *RAB3GAP1* gene with an extremely rare variant from a rural and poorly investigated region of Pakistan, missing in all the public databases. The identification of a rare causative variant in this study necessitates the investigation of more WARBM cases to identify yet undiscovered causative variants reflect on the actual frequency and spectrum of variants in the causative genes.

## Figure legends

**Figure: A)** Pedigrees of the family and **B)** Clinical phenotypes of the male (upper panel) and female patient (lower panel). **C)** MRI shows simplified pattern of sulci and gyri and narrow corpus callosum. **D)** Chromatographs of sequence analysis of *RAB3GAP1* of the patient and parents.

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## Ethical approval and consent to participate

Institutional Bioethical Committee (IBC) of Islamia College University Peshawar (Ref. No. 602/ORIC/ICP).

## Disclosure

The authors declare that they have no competing interests.

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## Availability of data and materials

The datasets used and analyzed supporting our findings are included in the main manuscript. The raw data during the current study is available to researchers on request from the corresponding author.

## Consent for publication

Informed written consent for publication of medical data and images was obtained from the legal guardian of family.

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